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Pseudomonas aeruginosa-induced lung injury: role of oxidative stress

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Pseudomonas aeruginosa is a Gram-negative pathogen that can cause lung injury in immunocompromised patients, primarily by inducing a release of host-derived mediators responsible for the influx of phagocytes to the lung. These phagocytes exert their antimicrobial actions by releasing toxic metabolites, including reactive oxygen species and proteases, which can also cause cell injury. This study was carried out to assess the pulmonary oxidant–antioxidant status of male adult Sprague–Dawley rats infected with different numbers of *P. aeruginosa* (10^4–10^7 cfu/animal). Intratracheal instillation of *P. aeruginosa* resulted in lung injury, as evidenced by increases in wet lung weight and decreases in the lung activities of angiotensin converting enzyme and alkaline phosphatase, enzymes localized primarily in pulmonary endothelial and alveolar type II epithelial cells, respectively. The *P. aeruginosa*-induced lung injury was directly related to the infiltration of neutrophils, as indicated by increases in myeloperoxidase activity. The challenge of animals with *P. aeruginosa* resulted in increases in lipid peroxidation and decreases in glutathione content, which were associated with the indices of lung injury and neutrophil infiltration. Such a challenge also resulted in weakening the antioxidant defence system, as evidenced by decreases in superoxide dismutase, catalase and glutathione peroxidase activities. These data suggest that changes in the pulmonary oxidant–antioxidant status may play an important role in the *P. aeruginosa*-induced lung injury.

Key words: *Pseudomonas aeruginosa*, reactive oxygen species, oxidative stress, antioxidants.

Introduction

Pseudomonas aeruginosa is a common respiratory-tract pathogen that is innocuous in healthy individuals, but can cause serious infection in immunocompromised patients or in patients with cystic fibrosis [1, 2]. *P. aeruginosa* infection may result in acute lung injury, primarily mediated by host-derived mediators, including cytokines and eicosanoids, resulting in a massive influx of phagocytes to the lung [3, 4]. These inflammatory cells are the first line of defence against invading microorganisms and are known to exert their antimicrobial actions by releasing reactive oxygen species, proteolytic enzymes and other toxic metabolites [3, 5, 6].

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Unfortunately, these toxic metabolites do not discriminate between microbial pathogens and host cells, and thus can injure cells that exist in close proximity to activated inflammatory cells [5, 6].

One of the adverse effects of microbial infection on the host is believed to be oxidative stress, normally demonstrated by an elevation in the cellular steady-state concentration of reactive oxygen species, such as superoxide anion, hydrogen peroxide and hydroxyl radical [7]. This condition can lead to the initiation of membrane lipid peroxidation and damage to DNA, proteins, nucleic acids and other macromolecules [7–9]. Generation of reactive oxygen species may not always lead to cellular injury, because of the presence of antioxidant defence systems including superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and glutathione peroxidase (GSH-Px) [10, 11]. However, when these defence systems are adversely affected or overwhelmed, such as in the case of acute oxidative stress, it can lead to changes in the structure and function of cellular components. These changes include an acceleration of membrane lipid peroxidation, DNA damage, depletion of intracellular ATP and reducing equivalents, and an increased formation of intracellular oxidized sulphydryls, which can contribute to cell death [7–9, 11].

Bacterial lipopolysaccharides or other bacterial by-products have been used to examine the oxidant–antioxidant status of lungs and other organs [10, 12, 13], but to our knowledge, no study has been conducted to systematically examine the effect of bacterial infection on the oxidant–antioxidant status of the lung. The objective of this study was to examine whether the oxidant–antioxidant balance was altered in the lungs of *P. aeruginosa*-infected animals. An imbalance in the oxidant–antioxidant status in infected lungs could contribute to the pathogenesis of lung injury. A better understanding of such an imbalance may shed light in developing therapeutic strategies to improve the outcome of infection-induced pulmonary injury.

In the present study, *P. aeruginosa*-induced oxidative stress was assessed by measuring changes in lipid peroxidation and glutathione content in the lung homogenates. The status of the antioxidant system was assessed by measuring the activities of SOD, CAT and GSH-Px. The extent of lung injury was determined by measuring the wet lung weights and the activities of angiotensin converting enzyme (ACE) [14] and alkaline phosphatase (AKP) [15] indices of capillary endothelial cell and alveolar type II epithelial cell injuries, respectively, while the inflammatory condition was monitored by measuring the activity of myeloperoxidase (MPO) and the pulmonary contents of proteases and chloramines [5, 6, 16].

Results

Wet lung weight and pulmonary ACE and AKP activities

The effect of intratracheally administered *P. aeruginosa* on wet lung weight is shown in Fig. 1(a). The weights of wet lungs were significantly increased in a bacterial concentration-dependent manner with an 83% increase observed 3 days after intratracheal administration of 10^7 cfu/animal. Since ACE and AKP enzymes have been used as markers of lung injury [14, 15], the effect of pulmonary infection on the activities of these enzymes was also measured. As shown in Fig. 1(b) and (c), instillation of 10^4 cfu of *P. aeruginosa* did not alter the status of these parameters to any significant extent, while instillation of 10^7 cfu resulted in dramatic decreases in both enzyme activities (69 and 53%, respectively).

Changes in pulmonary MPO activity and chloramine and protease contents

The infiltration and activation of neutrophils in the lungs of infected animals were assessed by measuring the activity of MPO and the pulmonary contents of proteases and chloramines [5, 6, 16]. As shown in Fig. 2(a), infection of animals with *P. aeruginosa* resulted in a bacterial concentration-dependent increase in pulmonary MPO activity, suggestive of neutrophil infiltration. More precisely, the increases in MPO activity ranged between two- and 25-fold after instillation of 10^4–10^7 cfu of *P. aeruginosa*, respectively. Also, infection of rats with the bacteria (10^4–10^7 cfu) resulted in significant increases in pulmonary protease (24–206%) and chloramine (70–628%) contents, suggestive of phagocyte activation.

Lipid peroxidation levels and GSH concentrations in the lung

Several studies have shown that lipid peroxidation is a possible mechanism of acute

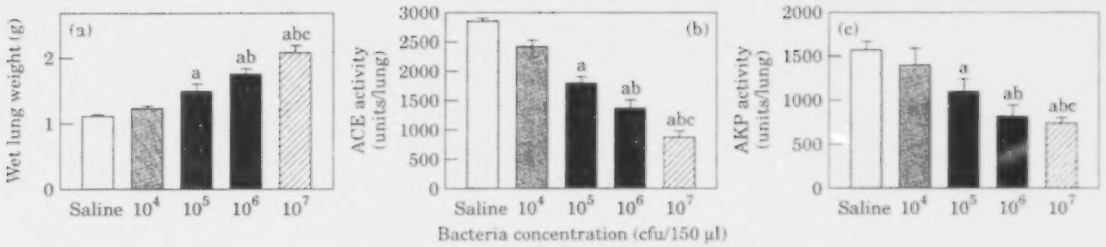

Figure 1. Effects of *P. aeruginosa* lung infection on pulmonary wet lung weight (a), ACE activity (b) and AKP activity (c). Each vertical bar represents the mean ± SE of six animals. a, significantly different ($P<0.05$) from the corresponding control value; b, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^5 cfu of *P. aeruginosa*; c, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^6 cfu of *P. aeruginosa*.

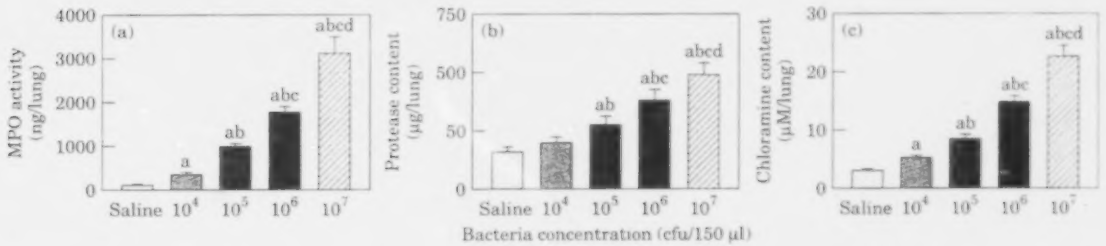

Figure 2. Effects of *P. aeruginosa* lung infection on pulmonary MPO (a), protease (b) and chloramine (c) contents. Each vertical bar represents the mean ± SE of six animals. a, significantly different ($P<0.05$) from the corresponding control value; b, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^4 cfu of *P. aeruginosa*; c, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^5 cfu of *P. aeruginosa*; d, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^6 cfu of *P. aeruginosa*.

oxidative-stress-induced lethal injury [8, 9]. Therefore, in this study, the levels of lipid peroxidation in lung homogenates of control and infected animals were also measured. Infection of animals with *P. aeruginosa* produced dramatic increases in lipid peroxidation levels, as shown in Fig. 3(a), which ranged between seven- and 33-fold after instillation of 10^4–10^7 cfu of *P. aeruginosa*, respectively.

Since a depletion of glutathione has been suggested to result in cellular injury, pulmonary glutathione levels of control and infected animals were measured [8, 9]. Infection of rats with *P. aeruginosa* resulted in a significant decrease in their lung GSH contents in a bacterial concentration-dependent manner, as shown in Fig. 3(b), with a 45% reduction after instillation of 10^7 cfu of *P. aeruginosa*.

Enzyme activities of lung SOD, CAT and GSH-Px

To assess the relative importance of the antioxidant system in *P. aeruginosa* infection, the effects of infection on lung SOD, CAT and GSH-Px activities were examined. Instillation of 10^4 cfu of *P. aeruginosa* did not alter the status of the antioxidant enzymes to any significant extent, as shown in Fig. 4; however, instillation of 10^5–10^7 cfu resulted in dramatic decreases in the activities of SOD (38–66%, respectively), CAT (43–70%, respectively) and GSH-Px (14–37%, respectively).

Plasma phospholipase A_2 concentrations

Phospholipase A_2 (PLA_2) is known to be involved in the inflammatory process by liberating

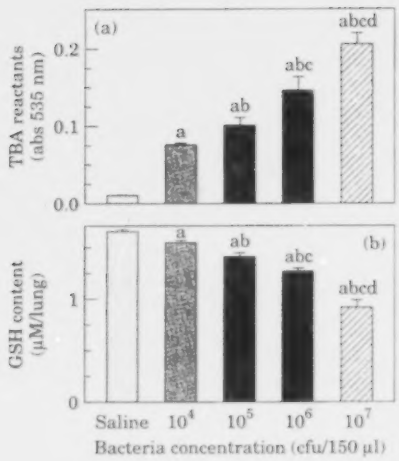

Figure 3. Effects of *P. aeruginosa* lung infection on pulmonary lipid peroxidation levels (a) and GSH content (b). Each vertical bar represents the mean ± SE of six animals. a, significantly different ($P<0.05$) from the corresponding control value; b, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^4 cfu of *P. aeruginosa*; c, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^5 cfu of *P. aeruginosa*; d, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^6 cfu of *P. aeruginosa*.

free arachidonic acid from membranes for the biosynthesis of pro-inflammatory mediators such as thromboxanes, prostaglandins and leukotrienes, potent chemotoctic agents for phagocytes [17, 18]. In the present study, the intratracheal instillation of *P. aeruginosa* resulted in significant increases in plasma PLA_2 concentrations and these changes correlated with the number of bacteria instilled (Fig. 5).

Discussion

The results of this study indicated that intratracheal instillation of *P. aeruginosa* in animals resulted in lung injury, as evidenced by increases in wet lung weights and changes in the biochemical indices of pulmonary injury, namely decreased ACE and AKP activities. Pulmonary capillary endothelial cells are known to be rich in ACE and a decrease in ACE activity has been shown to be a useful indicator of endothelial cell damage in the lung [14]. Alkaline phosphatase is an enzyme primarily localized in pulmonary alveolar type II epithelial cells [15], which are progenitors of type I cells and responsible for the production of surfactant [19]. Injury to the air–blood barrier and impairment of surfactant production in the lung can cause pulmonary edema [19, 20], as evidenced by the increases in lung weights. These findings are in agreement with results presented in other studies, where lung infection with *P. aeruginosa* in experimental animals and humans has been associated with lung injury and edema [2, 21, 22].

The exact mechanisms of lung injury observed following the intratracheal administration of *P. aeruginosa* remain to be elucidated. In our study, instillation of *P. aeruginosa* resulted in an accumulation of neutrophils in the lung, as suggested by increases in MPO activity. Neutrophils are known to migrate into tissues in response to chemotactic stimuli such as PLA_2, eicosanoids and tumor necrosis factor [3, 5, 17, 18]. Although

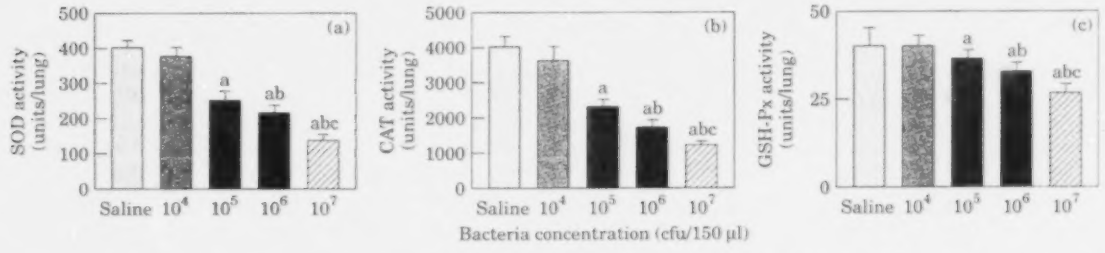

Figure 4. Effects of *P. aeruginosa* lung infection on pulmonary SOD (a), CAT (b) and GSH-Px (c) activities. Each vertical bar represents the mean ± SE of six animals. a, significantly different ($P<0.05$) from the corresponding control value; b, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^4 cfu of *P. aeruginosa*; c, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^5 cfu of *P. aeruginosa*.

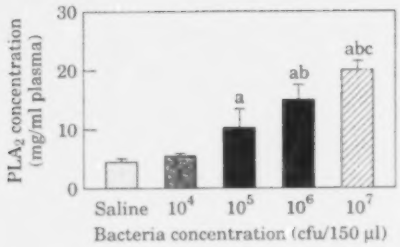

Figure 5. Effects of *P. aeruginosa* lung infection on pulmonary PLA_2 content. Each vertical bar represents the mean ± SE of six animals. a, significantly different ($P<0.05$) from the corresponding control value; b, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^5 cfu of *P. aeruginosa*; c, significantly different ($P<0.05$) from the corresponding value obtained from animals infected with 10^6 cfu of *P. aeruginosa*.

neutrophils have been ascribed as a first line of defence against microorganisms, their accumulation in the lung is an early step in the pathogenesis of acute lung injury [3, 5, 6]. This tissue injury is characterized by damages to the vascular endothelium and alveolar epithelium, as suggested in our study by decreases in pulmonary ACE and AKP activities.

An increase in peroxidation of membrane lipids and a decrease in GSH in the lungs of infected animals strongly suggest the involvement of oxidative stress-mediated mechanisms of injury [7–9]. Peroxidation of membrane lipids has been described in humans with sepsis or after administration of endotoxin and other bacterial byproducts in animal models [10, 12, 13]. Generally, lipid peroxidation is an irreversible chain reaction that occurs when polyunsaturated fatty acids react with reactive oxygen species, resulting in changes in the structure and function of cellular membranes, which may lead to increases in permeability and cell injury [7–9]. During the process of lipid peroxidation, the byproducts generated (i.e. molandialdehyde and lipid peroxides) may enhance the process of lipid peroxidation. The generation of lipid peroxidation products has been associated with a depletion in GSH levels [8, 23]. It has been established that GSH serves as a reductant in the metabolism of various hydroperoxides, generated following the peroxidation of membrane lipids, a reaction catalyzed by glutathione peroxidase [8, 23]. Overall, an increase in lipid peroxidation and a depletion of GSH in lung injury suggest that oxidative stress is involved.

The mechanism(s) of oxidative stress-mediated lung injury induced by *P. aeruginosa* cannot be delineated from the results of this study. Activated neutrophils and other phagocytes release reactive oxygen species, including superoxide anion and chloramines, responsible for killing bacteria, but also known to exert deleterious effects on lung structure and function [3, 5, 6]. This is consistent with the results of this study, where the increases in chloramine concentration, chloramines being long-lived oxidants released from neutrophils [5, 24], in lung homogenates were associated with increased MPO activity and other changes in oxidative stress indices. The role of reactive oxygen species in inducing injury to the lung and other tissues, as a result of an infection-induced inflammatory response, has been reported by other investigators who showed an increased oxidative stress in patients or experimental animals with pneumonia [25, 26], HIV [27] and malaria [28, 29].

Tissue injury via oxidative stress mechanisms is attributable not only to the overproduction of reactive oxygen species, but also to the status of the antioxidant defense system. It has been established that tissue injury via oxidative stress-mediated mechanisms occurs only when there is an imbalance between the oxidant–antioxidant status [11, 16]. The observed decreases in the antioxidant buffering capacity of the lung may increase the susceptibility of tissues against potential oxidative stresses. However, the mechanism by which *P. aeruginosa* infection depresses the antioxidant buffering capacity of the lung is not known. One possible explanation is that antioxidant enzymes may be partially inactivated by reactive oxygen species, proteases or other potentially toxic mediators associated with the inflammatory response to infection [30–32]. Another explanation may be that these antioxidant enzymes are released from injured cells into the general circulation, thus resulting in a reduction in the pulmonary antioxidant enzyme activities.

In addition to the potential injurious actions of reactive oxygen species, other metabolites released in the inflammatory process may contribute to tissue injury. In this study, the protease content in lung homogenates of infected animals was increased in a manner dependent on the bacterial concentration. Proteases have been shown to modify the extracellular matrix components and/or lung parenchymal cells, and play a central role in the lung by modifying

proteins that participate in the complement system, in coagulation, and other protein cascade systems [5, 6]. The increase in protease content might be caused by an increase in neutrophil accumulation in the lung. The results of this study, however, cannot exclude the possibility that an increase in protease content could also be due to the inactivation of protease inhibitors by reactive oxygen species. In any event, it has been reported that reactive oxygen species and proteases can work synergistically to enhance their injurious effects [24, 33].

The role of specific virulence factors from this organism on lung injury were not examined in the present study. Results from previous studies, however, have demonstrated that virulence factors, such as elastase and exotoxin A, could also augment the initial pulmonary injury. For example, elastase from *P. aeruginosa* seems to increase alveolar epithelial permeability by damaging the tight junction-associated proteins while exotoxin A, through its effect on protein synthesis, may render the cells unable to restore the junctional proteins [34, 35]. Therefore, increased epithelial permeability may facilitate the influx of neutrophils into the alveolar region, thus exacerbating the inflammatory response.

In summary, the results of this study indicated that lung infections with *P. aeruginosa* adversely affected the tissue oxidant–antioxidant status. Such an imbalance could increase the susceptibility of the lung to inflammation and supplementation with antioxidants should be considered as a part of therapy. Reports from previous studies have evaluated the effectiveness of antioxidants in the treatment of pulmonary viral infections and the results appear promising [36, 37]. However, it should be recognized that there is a possibility that the susceptibility of lung tissues to injurious agents may be enhanced following antioxidant supplementation, because antioxidants may also protect bacteria against the toxic actions of reactive oxygen species released by phagocytes. Experiments are currently underway to determine whether the use of antioxidants in preserving the integrity of the lung is beneficial or detrimental.

Materials and Methods

Bacterial strain

P. aeruginosa (ATCC 25619) (PML Microbiologicals, Mississauga, ON, Canada) was stored at −70°C in trypticase soy broth supplemented with 10% (v/v) glycerol. For experimentation, this strain was inoculated onto blood agar plates (PML Microbiologicals, Mississauga, ON, Canada) and incubated for 18 h at 37°C.

Chemicals

All chemicals and reagents used were purchased either from Sigma Chemical Co. (St. Louis, MO, U.S.A.) or BDH (Toronto, Ontario, Canada).

Animals

Male Sprague–Dawley rats, (250–275 g body weight), were purchased from Charles River Canada, Inc. (St Constant, Quebec, Canada). All animals were housed in stainless-steel cages with free access to pelleted purina laboratory chow and tap water. The animals were exposed to alternate cycles of 12 h light and darkness. Animals used in this research were cared for in accordance with the principles contained in the Guide to the Care and Use of Experimental Animals, recommended by the Canadian Council on Animal Care. The experimental protocol was approved by the institutional animal care committee.

Treatment of animals

Rats were anaesthetized by an intraperitoneal injection of a mixture of 50 mg/kg ketamine (Ketalar, Parke-Davis, Scarborough, Ontario, Canada) and 20 mg/kg xylazine (Rompun, Bayvet, Etobicoke, Ontario, Canada). The endotracheal intubation technique described by Suntres and Shek [38] was adapted for administering the bacteria inoculum. Animals were challenged with different concentrations of bacteria (10^4–10^7 cfu/150 µL) and killed 72 h later.

Tissue preparation

Lungs were removed from animals immediately after decapitation and rinsed in ice-cold saline to remove excess blood. All subsequent steps were carried out at 0–4°C. Following rinsing, lungs were quickly blotted, weighed and finely minced. Approximately 1 g of lung tissue was

homogenized in a sufficient volume of ice-cold 50 mM potassium phosphate buffer, pH 7.4, to produce a 20% homogenate.

Enzyme measurements

Activities of ACE and AKP in lung homogenates were determined, as previously described [39]. The activity of MPO in sonicated whole lung homogenates was carried out by using a kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturers directions. The activities of SOD, CAT and GSH-Px were estimated spectrophotometrically as previously described [40]. Phospholipase A_2 activity was estimated by using a specific enzyme-linked immunosorbent assay kit (Boehringer Mannheim Canada, Laval, Quebec, Canada) according to the manufacturers directions. The protease content in lungs was assayed by a QuantiCleave protease assay kit (Pierce Chemical Co., Rockford, IL, U.S.A.) which is based on the cleavage of succinylated casein by proteases in the sample.

Determinations of pulmonary lipid peroxidation and reduced glutathione content

Determination of lipid peroxidation was carried out by using a kit (R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturers directions. This procedure allows the measurement of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) concentrations. Reduced GSH, more precisely non-protein sulphydryl, concentrations in pulmonary homogenates, were determined as described by Suntres and Shek [40].

Determination of chloramine concentration

Chloramine concentrations in pulmonary homogenates were determined by colorimetric measurement of the triiodide ion formed by the oxidation of potassium iodide [41].

Data analysis

The results are expressed as means ± SEM obtained from three separate experiments. Comparisons among groups were evaluated by one-way analysis of variance (ANOVA) with a Newman–Keuls test of multiple comparisons. A P value of 0.05 or less was considered significant [42].

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